Antisense therapeutics: is it as simple as complementary base recognition?

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Antisense oligonucleotides provide a simple and efficient approach for developing target-selective drugs because they can modulate gene expression sequence-specifically. Antisense oligonucleotides have also become efficient molecular biological tools to investigate the function of any protein in the cell. As the application of antisense oligonucleotides has expanded, multiple mechanisms of oligonucleotides have been characterized that impede their routine use. Here, we discuss different mechanisms of action of oligonucleotides and the possible ways of minimizing antisense-related effects to improve their specificity.

SINCE the first report of the use of antisense oligodeoxynucleotides to inhibit *Rous sarcoma* virus gene expression[1], there has been tremendous progress in the understanding and application of antisense oligodeoxynucleotides. Simplicity, rational design, the inexpensive availability of synthetic oligodeoxynucleotides and developments in human genome sequencing have contributed to this progress. In addition, antisense technology has become an essential laboratory tool to study and understand the function of any newly discovered genes in recent years. In principle, the antisense approach should allow the design of drugs that specifically intervene with the expression of any gene whose sequence is known.

Chemical modification of the natural phosphodiester backbone is necessary to prevent its rapid degradation by ubiquitous nucleases. Of all the chemical modifications developed, **phosphorothioate oligodeoxynucleotides** (**PS-oligonucleotides**) are the most extensively studied analogs of the phosphodiester oligonucleotides and several are currently under evaluation for their therapeutic potential in human clinical trials. In **PS-oligonucleotides**, one of the non-bridging oxygens of the phosphate is replaced with a sulfur in order to prevent rapid degradation by nucleases (Fig. 1). In addition to nuclease resistance, **PS-oligonucleotides** possess important properties such as binding affinity to the target (mRNA), cellular uptake, aqueous solubility and the ability to activate **RNase H**, which is required for antisense activity[2,3]. As a result of these inherent favorable properties, **PS-oligonucleotides** have become the choice as the first generation of antisense molecules in hundreds of studies in cell cultures and animal models[2,3]. Recently, a **PS-oligonucleotide** targeted to human cytomegalovirus (CMV) has been approved for the treatment of CMV-induced retinitis[4]. Many other **antisense oligonucleotides** are at various stages of clinical development (Table 1).

As the number of reports of the use of **PS-oligonucleotides** in the literature increased, it became evident that the use of **PS-oligonucleotides** as antisense agents might not be as simple as initially expected[2,3,5]. The effects observed in many studies could be attributed to the antisense mechanism, but the effects observed in others could not[6,7]. These non-antisense effects could be the result of the presence of certain sequence motifs and/or secondary structures (due to self-complementarity) in the **PS-oligonucleotides** or they could be related to their polyanionic nature. These other modes of action of **PS-oligonucleotides** often overlapped with the mechanism of action and specificity of **antisense oligonucleotides**. It is now becoming evident that such defined factors can directly or indirectly influence the specificity and mechanism of action of **antisense oligonucleotides**.

In this article, the different mechanisms of action of **PS-oligonucleotides** that confuse the understanding of antisense effects are discussed. In addition, methods for the reduction or minimization of these complications by implementation and adherence to certain guidelines for the design and study of **antisense oligonucleotides** *in vitro* and *in vivo* are described. These descriptions cover not only the development of therapeutics, but also functional genomics applications, such as elucidating the biological function of any newly discovered gene by inhibiting its translation.

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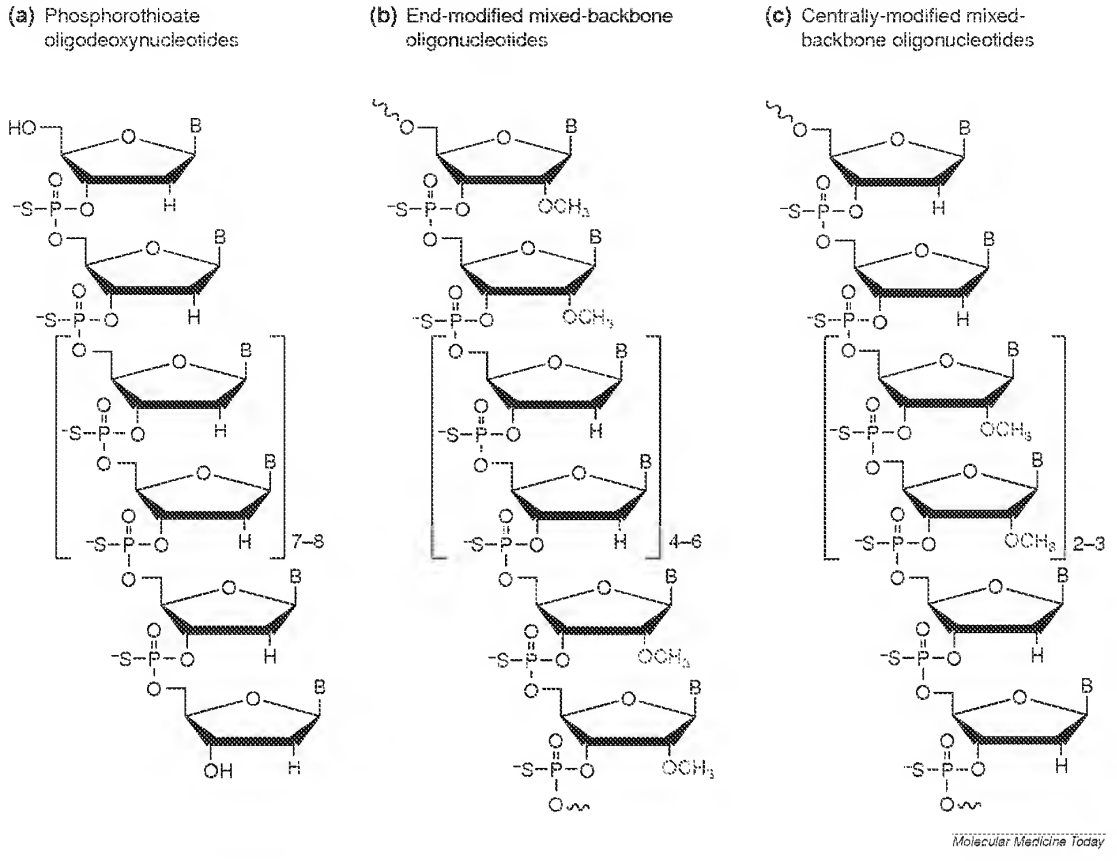

Figure 1. Structures of phosphorothioate (**a**), end-modified mixed-backbone (**b**), and centrally-modified mixed-backbone oligonucleotides (**c**). In (**b**) and (**c**) only 2′-O-methylribonucleotide modification is shown in **MBOs**. Any chemical modification that reduces polyanion-related side effects and increases antisense specificity can be used in both the end- and centrally-modified **MBOs**. The advantage of **MBOs** is that, although they retain the advantage of **PS-oligonucleotides** (**RNase H** activation), the inherent side effects can be minimized. The placement of methylphosphonate linkages (not shown in figure) at the ends reduces the overall polyanionic-related side effects, and increases the *in vivo* stability by protecting both the ends of the **PS-oligonucleotide** from digestion by nucleases. Similarly, placement of 2′-O-methyl-ribonucleotide segments provides increased affinity to the target mRNA and *in vivo* stability. Placement of modified segments in the center provides a handle on modulating the rate of degradation, the nature of metabolites being generated *in vivo* and the elimination of those metabolites. The modification that confers higher stability against nuclease digestion provides two advantages: longer duration of action to enable less frequent dosing and the presence of fewer degradation metabolites to decrease the possible side effects from such metabolites.

Mechanisms of action of PS-oligonucleotides

Theoretically, it is very simple to design oligonucleotides to inhibit the translation of encoded proteins by the antisense mechanism. In principle, an **antisense oligonucleotide** is designed to inhibit expression of specific unwanted protein by hybridizing to the target mRNA through Watson–Crick complementary base recognition, thereby physically blocking the ribosomal machinery and/or activating endogenous **RNase H** that cleaves the mRNA at the duplex site. There are numerous examples in which **PS-oligonucleotides** of varying lengths and base compositions have been employed to inhibit the translation of cellular or foreign genes by an antisense mechanism[7–11].

A major question that remains to be answered, however, is whether the inhibition of expressed protein or the subsequent biological effects observed are the result of a *bona fide* antisense mechanism. Detailed studies of the impact of **PS-oligonucleotide** sequence on their mechanism of action and specificity clearly suggest that sequence is a critical factor for many **PS-oligonucleotides**.

CpG motifs and immunostimulation

PS-oligonucleotides containing CpG motifs have immunostimulatory activity[12–15]: they induce many cytokines, including IL-12, IL-6, IFN-γ, TNF-α and chemokines[16,17]. Their immunostimulatory properties

Table 1. Oligonucleotides in clinical trials[a]

Molecular target	Sequence[b]	Disease target	Route of delivery	Status
Oncological and hematological diseases				
bcl-2	5'-TCTCCCAGCGTGCGCCAT-3'	Prostate; non-Hodgkin's lymphoma	Systemic, sc	Phase II/IIa
bcr-abl	5'-CGCTGAAGGGCTTCTTCCTTATTGAT-3'	CML, advanced phase	*Ex vivo* purging	Pilot
	5'-CGCTGAAGGGCTTTTGAACTGTGCTT-3'	CML, blast crisis	Systemic, iv	Pilot
c-myb	5'-TATGCTGTGCCGGGGTCTTCGGGC-3'	CML, blast crisis, refractory leukemia	Systemic, iv	Phase I
		CML, chronic/accelerated phase	*Ex vivo* purging	Pilot
c-myc	5'-GCTAACGTTGAGGGGCAT-3'	Restenosis	Systemic, iv	Withdrawn
c-raf	5'-TCCCGCCTGTGACATGCATT-3'	Prostate, breast, ovarian, pancreas, colon, lung	Systemic, iv	Phase II
DNA metase	Not known	Solid tumors	Systemic, iv	Phase I
Ha-ras	5'-GGGACTCCTCGCTACTGCCT-3'	Solid tumors	Systemic, iv	Phase I
ICAM-1	5'-GCCCAAGCTGGCATCCGTCA-3'	Crohn's disease, psoriasis, rheumatoid arthritis, ulcerative colitis, tissue rejection after organ transplantation	Systemic, iv	Phase II
p53	5'-CCCTGCTCCCCCTGCTCC-3'	AML and myelodysplastic syndrome, refractory or relapsed	Systemic, iv	Phase I
		AML and myelodysplastic syndrome	*Ex vivo* purging	Pilot
PKA-RIα	5'-**GCGU**GCCTCCTCA**CUGGC**-3'	Solid tumors	Systemic, iv	Phase II
PKC-α	5'-GTTCTCGCTGGTGAGTTTCA-3'	Ovarian, prostate, breast, brain, lung, colon, melanoma	Systemic, iv	Phase II
Viral diseases				
CMV	5'-GCGTTTGCTCTTCTTCTTGCG-3'	CMV-induced retinitis	Local, intravitreal	Approved
CMV	5'-**UGGGGCTTACGTTGCGAACA**-3'	CMV-induced retinitis	Systemic, iv	Phase II
HIV-1	5'-CTCTCGCACCCATCTCTCTCCTTCT-3'	HIV-1–AIDS	Systemic, iv	Withdrawn
HPV	5'-TTGCTTCCATCTTCCTCGTC-3'	Genital warts	Local, id	Withdrawn

[a]Abbreviations: AML, acute myeloid leukemia; CML, chronic myeloid leukemia; CMV, cytomegalovirus; HPV, human papilloma virus; ICAM, intercellular adhesion molecule; id, intradermal; iv, intravenous; metase, methyl transferase; PKA, protein kinase A; PKC, protein kinase C; RIα, regulatory subunit Iα; sc, subcutaneous.

[b]All the sequences contain phosphorothioate internucleotide linkages; plain and bold letters indicate deoxy- and 2'-O-methyl-ribonucleosides, respectively.

depend on the sequence, base composition and the position of the CpG motif in the sequence. **PS-oligonucleotides** containing CpG motifs might therefore have immunostimulatory properties in addition to the antisense function that they were designed for. For example, two 29-mers of the same base composition, except that one has a CG-motif and the other has a GC-motif, showed sequence-specific non-antisense mediated antiviral activity (Fig. 2a). Currently, **PS-oligonucleotides** containing CpG motifs are being explored as immunomodulators in antiviral, antibacterial, anticancer and anti-inflammatory therapies[18].

Is it possible that the nucleotide sequence of **PS-oligonucleotides** also has an impact on the observed side effects *in vivo*? For example, two **PS-oligonucleotides** of the same base composition but with different nucleotide motifs caused similar side effects in three different mouse models, but the severity of the side effects was sequence dependent (Fig. 2b–e). This example further indicates that two **PS-oligonucleotides** of the same length and base composition can behave differently *in vivo*. In addition, sequence independent side effects, such as complement activation and prolongation of **activated partial thromboplastin time** (**aPTT**), were also observed with the **PS-oligonucleotides**. From these two examples and from other studies reported in the literature, it is clear that if a selected **antisense oligonucleotide** sequence has a CpG motif, extreme care must be taken in establishing its specificity of antisense activity.

Secondary structures interfere with the antisense mechanism

The presence of secondary structures can also interfere with antisense activity by allowing the oligonucleotides to bind to unintended protein targets. Double-stranded **PS-oligonucleotides** that contain a *cis*-transcription recognition sequence bind sequence-specifically to transcription factors competitively and interfere with transcription. This is

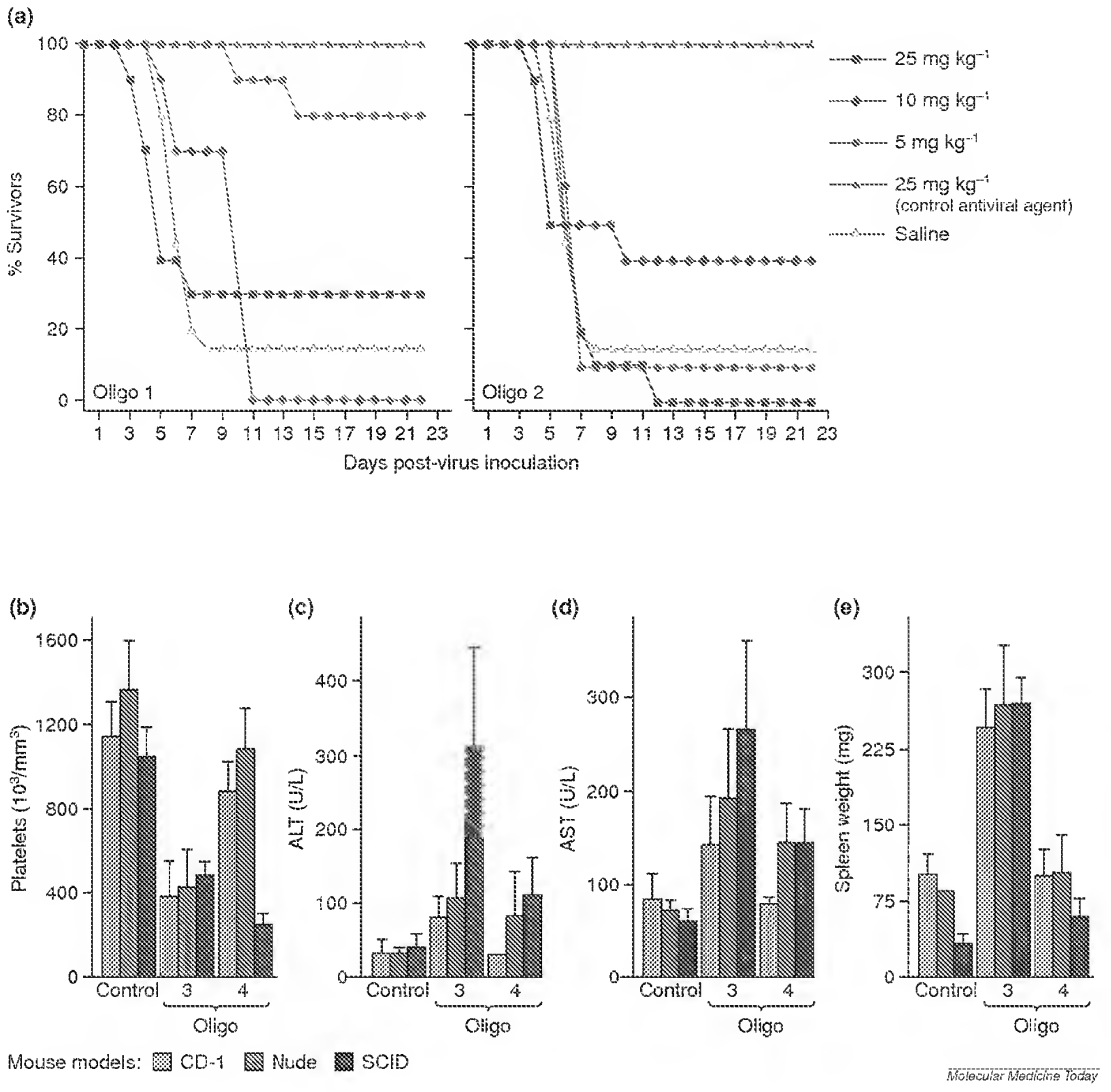

Figure 2 (**a**) The effect of **PS-oligonucleotides** 1 (oligo 1) (5′-CCATGACGTTCCTGATGCTTTTTGGGGG-3′) and oligo 2 (5′-TCCATGAGCTTCCTGATGCTTTTTGGGGG-3′) at 5, 10 and 25 mg kg^{-1} doses on CMV replication. A control antiviral agent, 3,5-dihydroxyphenylglycine (DHPG), was also used. Neither oligo 1 nor oligo 2 is complementary to murine-CMV RNA. The inhibition of CMV observed in this study was the result of immune stimulation rather than an antisense mechanism. At lower doses, protection of CMV-infected mice was observed with oligo 1. At higher doses, no protection was observed, probably because of hyper-stimulation of the immune system, which also resulted in increased toxicity. Analysis of serum showed an increase in IL-12 levels with oligo 1 compared to control (saline) or oligo 2 treated mice. (**b–e**) Side effects of oligo 3 (5′-TCGTCGCTGTCTCCGCTTCTTCTTGCC-3′) and oligo 4 (5′-TGCTGCCTGTCTGCCCTTCTTCTTGCC-3′) in CD-1, nude and SCID mouse models. Oligo 3 is complementary to the *rev* gene of HIV-1[13]. Oligo 3 caused significant changes in platelet count (**b**) and levels of transaminases, alanine aminotransferase (ALT) (**c**) and aspartate aminotransferase (AST) (**d**) in all three mouse models. Oligo 3 caused a greater increase in spleen enlargement (**e**) in all three mouse models than did oligo 4. In general, oligo 4 had less severe effects on the parameters studied. Examination of kidney, liver and spleen of the three mouse models for histopathology showed more pronounced reticuloendothelial cell hyperplasia with oligo 3 than oligo 4. In addition, hematopoetic cell proliferation was more pronounced in spleens of the three mouse models with oligo 3 than those with oligo 4. The side effects observed could be the result of immune-stimulatory properties of **PS-oligonucleotides** and might involve different pathways in these mouse models. Further studies are needed to characterize the mechanisms involved in inducing these side effects. It is important to note that, in several studies, nude and SCID mice are used as models to evaluate anti-tumor, antiviral or antibacterial activity. Factors responsible for side effects, directly or indirectly, might interfere with the mechanism of action of antisense or control **PS-oligonucleotides**

generally called the '**decoy**' mechanism of inhibition and it is different from antisense action. There have been several examples of the use of oligonucleotide-derived sequences as **decoys**[19–21].

Single-stranded oligonucleotides called **aptamers** are used to inhibit specific proteins in a sequence-specific manner. Examples of **aptamer** oligonucleotides include inhibitors of thrombin and HIV-1 integrase[22,23]. It is important to note that although the sequence of a **PS-oligonucleotide** is important in exerting non-specific activity, its internucleotide linkages, due to its polyanionic nature, also show sequence-independent side effects[24,25].

Optimal design of antisense oligonucleotides

It is clear that **PS-oligonucleotides** of varying sequences, lengths and base compositions could exert biological activities by many mechanisms. It is always possible that, although a unique structure or motif is required for non-antisense mechanism, the presence of these motifs in an **antisense oligonucleotide** or its control **PS-oligonucleotides** could interfere with the mechanism of action and specificity.

Therefore, **antisense oligonucleotide** design is not as simple as probe design and several points must be considered in the design and study of **antisense oligonucleotides**. A number of lessons have been learned from the **antisense oligonucleotide** studies in the past few years, and several chemical strategies (Fig. 3) have been developed to minimize non-antisense related effects of **PS-oligonucleotides**[2,3].

If proper design precautions, suitable chemical modifications and appropriate control sequences are not selected, **PS-oligonucleotides** with specific sequence motifs or secondary structures could influence the outcome of the experimental results through non-antisense mechanisms. Adoption and application of these modifications for **antisense oligonucleotide** design would be of particular interest to those who use antisense technology for elucidating the functions of newly discovered genes.

Target site selection

The initial step in selecting an **antisense oligonucleotide** is to choose an appropriate target sequence on the mRNA molecule. Antisense

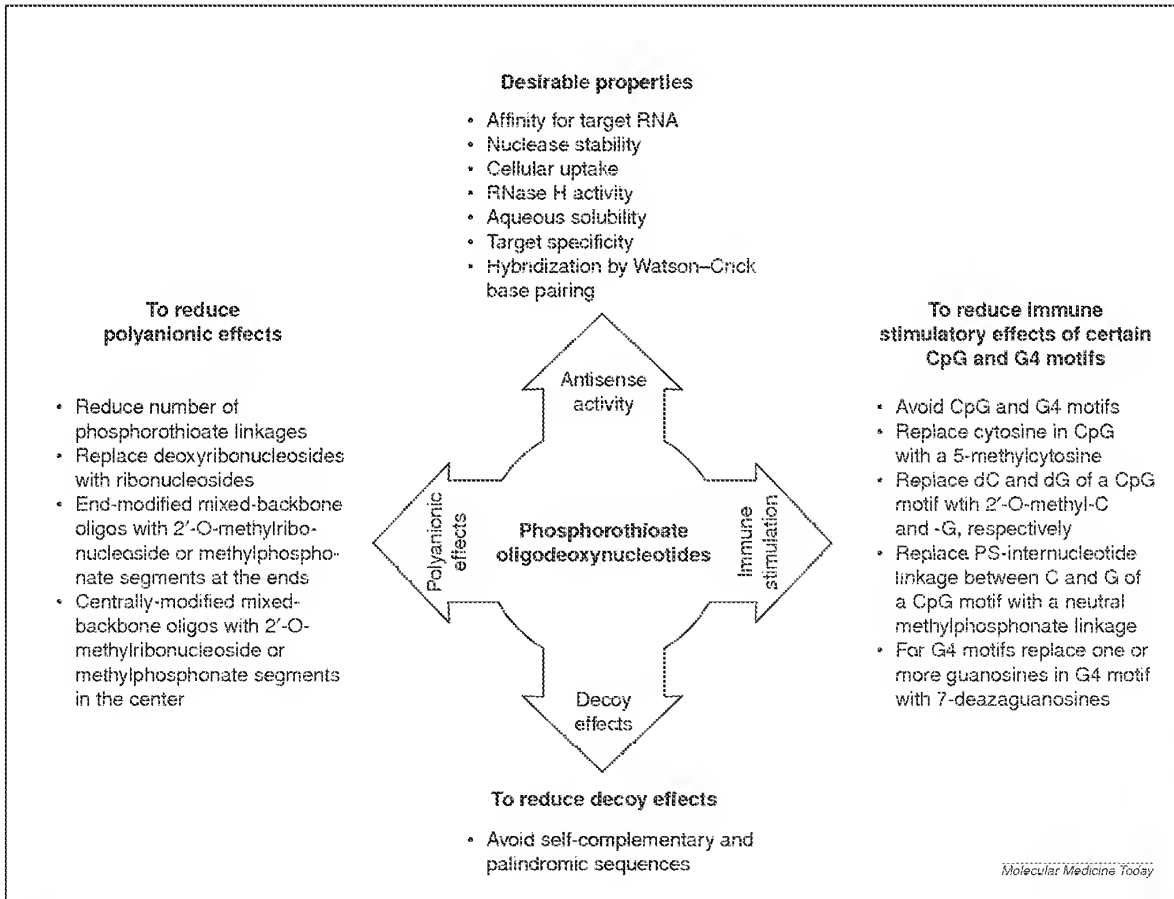

Figure 3. Factors that affect the specificity of antisense oligonucleotides and the chemical strategies that have been developed to reduce these non-antisense-related effects in order to improve specificity, pharmacokinetic and safety profiles.

Glossary

Activated partial thromboplastin time (aPTT) – An *in vitro* measurement to determine the prolongation of blood coagulation by coagulation inhibitors.

Antisense oligonucleotide – A synthetic oligonucleotide that is complementary to a portion of the targeted mRNA. It binds to the mRNA and arrests translation by physical blockade of ribosomal machinery and/or by activation of endogenous **RNase H**.

Aptamer – A single-stranded oligonucleotide that binds sequence-specifically to a protein and inhibits its function. An **aptamer** might adopt a specific structure in order to bind to the protein.

Decoy – A linear or hairpin duplex oligonucleotide that captures a transcription factor by competitive sequence-specific binding and inhibits transcription of a specific gene.

Hyper-structure-forming sequences – The G-rich sequences (containing three or more guanosines in a row) that form Hoogsteen hydrogen bonded structures involving guanines in four or more strands.

Mixed-backbone oligonucleotides (MBOs) – Oligonucleotides that are synthesized with more than one modification in order to improve biophysical, biochemical, pharmacokinetic or safety profiles.

Phosphorothioate oligodeoxynucleotides (PS-oligonucleotides) – Oligodeoxynucleotides in which one of the non-bridging oxygens on the phosphate of the natural phosphodiester backbone is replaced with a sulfur to make them nuclease resistant.

RNase H – A ribonuclease that specifically recognizes an RNA–DNA heteroduplex and cleaves the RNA strand of the heteroduplex.

5′- and 3′-untranslated regions (UTRs) – The nucleotide sequences that are present before a start codon and after a termination codon, respectively, in an mRNA and are not translated into protein.

technology has been hampered to some extent by limited knowledge as to the base-pairing accessibility of mRNA target sites *in vivo*. Although a number of models that predict RNA folding are available, their usefulness for predicting the most plausible *in vivo* RNA structure is limited[26]. Alternatively, *in vitro* methods can be used to test the accessibility of mRNA sites by oligonucleotides, but this has also met with limited success[27]. It is considered preferable, therefore, to screen a number of oligonucleotides that encompass different regions on RNA to identify a set of optimal target sites, including the **5′- and 3′-untranslated regions (UTRs)**, initiation codon site, coding region and intron–exon junctions. Oligonucleotides that have been targeted to the translation initiation codon region of mRNA are generally believed to be more potent than those targeted to other regions. Our experience, however, shows that it is difficult to find a 20-nucleotide site that includes the initiation codon and satisfies all the criteria discussed in this review for optimal **antisense oligonucleotide** design.

It has recently been shown that sites containing GGGA sequence motifs on mRNA are more accessible to **antisense oligonucleotides** than are other sites[28]. Although it is interesting, it is not possible to generalize this concept for targets other than those examined until further evidence emerges with a number of other targets.

Choice of oligonucleotide sequences

The affinity of an oligonucleotide for its target RNA varies significantly depending on base composition and sequence[29,30]. Therefore, the antisense activity of a selected oligonucleotide is influenced both by its base composition and by its sequence. Oligonucleotides that contain certain sequence motifs, such as CpG (Refs 12,14,15) and GGGG (**hyper-structure-forming sequences**)[31,32], induce cell proliferation and immune responses. G-rich oligonucleotides also have different cellular uptake, tissue distribution, pharmacokinetics and *in vivo* disposition from those oligonucleotides that do not contain four or more adjacent guanosines[31]. Antisense **PS-oligonucleotides** containing CpG and G-rich motifs increase the possibility of exhibiting activities by non-antisense mechanisms. Hence, it is appropriate to avoid sequences containing these motifs for antisense uses.

If an **antisense oligonucleotide** possesses self-complementarity or a palindromic sequence, it can form stable secondary structures, such as short linear duplexes or hairpins (Fig. 4). In such cases, secondary structure formation competes for binding to the target mRNA. In addition, these secondary structures can serve as **decoys** by binding to cellular factors, thereby inhibiting or inducing the functions of non-targeted genes, which could directly or indirectly alter the function of the gene being studied. A number of software packages are currently available for screening oligonucleotides for the formation of secondary structures involving traditional Watson–Crick base pairing rules. However, these packages are not designed to predict non-traditional structures, such as quadruplexes involving G-rich motifs, GA base pairs, parallel-stranded structures and the possible structures formed by chemically modified nucleotides. In these cases, non-denaturing gel electrophoresis or UV thermal denaturation methods can be used to examine the possible secondary structure formation by **antisense oligonucleotides** alone (Fig. 4).

Choice of chemical modification

The sequence-unrelated side effects associated with **PS-oligonucleotides**[12,33] have led to attempts to improve specificity and thereby reduce side effects. The PS-backbone, in association with deoxyribonucleotides, is responsible for polyanion-related effects, such as prolongation of **aPTT** and complement activation[34]. PS-oligoribonucleotides, PS-2′-O-methyl-oligoribonucleotides and PS-2′-5′-linked-oligoribonucleotides (see Fig. 5 for chemical structures) have lower or negligible effects on prolongation of **aPTT** and complement activation[34].

Methylphosphonate oligonucleotides show less severe polyanion-related effects because they have reduced negative charge[15,33,35]. Unfortunately, methylphosphonates and PS-oligoribonucleotides do not activate **RNase H** upon binding to the RNA target, an important property critical for the antisense mechanism. In addition, aqueous solubility of methylphosphonate oligonucleotides containing more than 12 nucleotides becomes a limiting factor. We have therefore attempted to use a combination of these modifications with **PS-oligonucleotides** to develop **antisense oligonucleotides** that have all the required properties for antisense activity while minimizing the polyanion-related effects[3]. These oligonucleotides are referred to as **mixed-backbone oligonucleotides (MBOs)** (Fig. 1).

MBOs containing segments of **PS-oligonucleotide** and other modified oligonucleotide segments have emerged as second-generation **antisense oligonucleotides**[2,3,36–38]. A number of modifications, combined with the **PS-oligonucleotide** backbone, have been studied[3], but two modifications – PS-2′-O-methyl(alkyl)ribonucleotides and methylphosphonate oligonucleotides – stand out because they reduce

several polyanion- and non-antisense-related side effects of **PS-oligonucleotides**[2,3]. Both modifications reduce protein binding and non-antisense effects related to the polyanionic nature of the **PS-oligonucleotides**[33,37–39]. These modifications can be incorporated at the 3′-end or at both the 3′- and the 5′-ends of a **PS-oligonucleotide** to produce 'end-modified **MBOs**', or incorporated in the center of the **PS-oligonucleotide** to produce 'centrally modified **MBOs**'.

End-modified **MBOs** have shown improved specificity, biological activity, *in vivo* stability, pharmacokinetic and safety profiles over **PS-oligonucleotides**[35–42]. Importantly, end-modified **MBOs** permit oral and colorectal administration of **antisense oligonucleotides** as a result of their increased *in vivo* metabolic stability[43]. In addition, end-modified **MBOs** have lower polyanion-related effects, such as complement activation and prolongation of **aPTT**, than do **PS-oligonucleotides**[33].

Centrally-modified **MBOs** contain a modified oligonucleotide segment placed in the center of a **PS-oligonucleotide**[38]. These **MBOs** show increased binding affinity to the target, increased **RNase H** activation, and consequently rapid degradation of RNA compared with end-modified **MBOs**. Centrally modified **MBOs** permit reduction of contiguous **PS-oligonucleotide** length, thereby determining their length-dependent polyanionic effects. These **MBOs** show improved pharmacokinetic and safety profiles and maintain biological activity similar to that of **PS-oligonucleotides**[38].

The purity of **PS-oligonucleotides** also affects their biological activity[44]. As a result of recent developments in synthetic, purification and analytical methodologies, it is now possible to obtain consistently pure **PS-oligonucleotides**. However, it is important for the laboratories that obtain oligonucleotides from commercial suppliers to check purity levels constantly as these vary from vendor to vendor and from batch to batch.

Avoiding CpG motifs in an antisense sequence reduces non-antisense-related activity considerably. However, if a CpG motif is absolutely necessary for antisense activity, the non-antisense-related effects of CpG motifs can be reduced by several chemical modifications (Fig. 3). These modifications include: (1) replacement of the cytosine base in the CpG motif with a 5-methylcytosine base; (2) replacement of the phosphorothioate linkage between C and G of the CpG motif with a methylphosphonate linkage; and (3) replacement of the d(CpG) motif with 2′-O-methylribonucleosides. We have studied a number of **PS-oligonucleotides** that contain CpG motifs with and without the above modifications in mice and rats and have found them to have significantly reduced side effects[15,40,41].

As for G-rich motifs, no known chemical modification (except replacement of Gs in the G-rich site with 7-deazaguannes) prevents hyperstructure formation. It is appropriate, therefore, to avoid sequences that

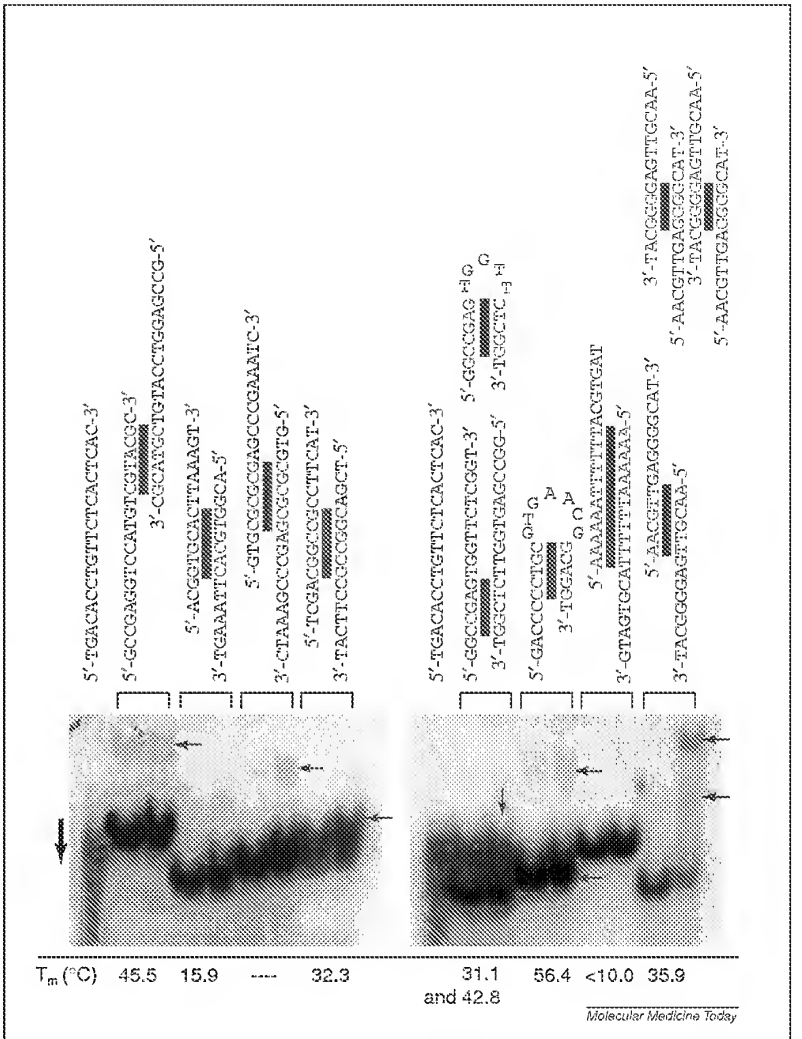

Figure 4. An autoradiogram showing structures formed by a number of **PS-oligonucleotide** sequences that have been reported in the recent literature. Sequences and possible structures formed by these oligonucleotides are shown on top of the gel. For each oligonucleotide there are two lanes except for the oligonucleotide shown as single strand (first lane in both the gels). These left and right lanes represent samples loaded with formamide after heating to 95°C and snap-cooling or loaded with glycerol buffer without heating, respectively. Bands indicated with an arrow are the result of secondary structure formation by these **PS-oligonucleotides**. The thick arrow beside the gel photograph indicates the direction of gel mobility. The UV thermal melting temperature (T_m) of each **PS-oligonucleotide** (measured in 100 mM NaCl, 10 mM sodium phosphate buffer pH 7.4 at a strand concentration of 4 μM in 1 ml) is shown below the autoradiogram. The observation of two T_m values for one oligonucleotide revealed the presence of more than one structure (although this was not distinguishable on the gel). An oligonucleotide that revealed no T_m value indicated the absence of secondary structure formation.

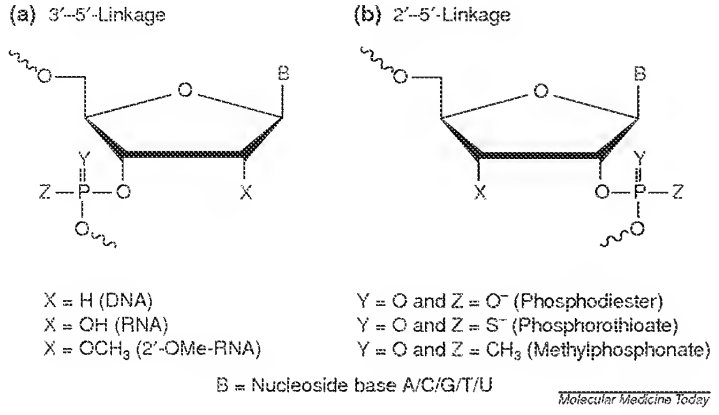

Figure 6. Structures elucidating (a) 3′–5′- and (b) 2′–5′-linkages and some of the chemical modifications discussed in this review.

contain four or more Gs (sometimes three) in a stretch. Hyper-structure formation by oligonucleotides that contain three or four adjacent Gs can be ascertained by examining them on non-denaturing polyacrylamide gels under physiological conditions because hyper-structure formation is also dependent on the flanking sequences (Fig. 4). In addition, synthesis and purification of G-rich oligonucleotides is complex and is not reproducible[45].

Minimization of protein binding and other competing factors

PS-oligonucleotides bind to proteins through sequence-specific, structure-specific and non-specific interactions. As a result of the polyanionic nature of the internucleotide phosphorothioate backbone, **PS-oligonucleotides** interact non-specifically with a number of proteins and enzymes *in vitro* in a sequence-independent, but length-dependent, manner[39,46–48]. **PS-oligonucleotides** inhibit the activities of several enzymes, including DNA polymerases, growth factors, protein kinase C, HIV-gp120, recombinant soluble CD4, reverse transcriptase, **RNase H** and RNase L in a dose-dependent fashion in *in vitro* studies, but at much higher concentrations than those required for antisense activity[39,46,47]. However, the *in vivo* biological relevance of inhibition of these enzymes has not yet been established. Note that these effects are mechanistically distinct from **decoy** and **aptamer** effects.

In addition, **PS-oligonucleotides** bind to a number of plasma/serum-proteins, notably albumin[33,39,48]. The binding affinity of **PS-oligonucleotides** for plasma/serum proteins has been shown to be in the order fibrinogen>γ-globulins>albumin[33,34,39]. As shown previously, **PS-oligonucleotides** demonstrate prolongation of **aPTT** and complement activation in a dose- and length-dependent, but sequence-independent, manner *in vivo* and *in vitro*[32–34,37,38,45,49]. It has recently been shown that **PS-oligonucleotides** inhibit the intrinsic tenase complex (factor IXa_β, factor VIIIa, phospholipid and calcium) in the blood coagulation system[50]. *In vivo* and *in vitro* studies have demonstrated that small molecules, such as aspirin, compete effectively for serum-protein binding and alter pharmacokinetic and tissue distribution profiles of **PS-oligonucleotides**[39,48]. These polyanion-related and protein binding-related side effects can be significantly minimized by maintaining low plasma concentrations by slow intravenous infusion as currently employed in clinical trials[32], by using formulations[33], or by incorporating 2′-O-alkyl(methyl)ribonucleosides or methylphosphonate linkages into **PS-oligonucleotides** as in the case of **MBOs** (Refs 33–35,37,38) (Fig. 3).

Control sequences

Although the antisense field has progressed to clinical trials, there is no consensus on what kind of controls to include in antisense experiments, mainly because of the limited knowledge of the biological effects of possible nucleotide motifs. So far, only the biological consequences of certain motifs, such as CpG and G4s, are known. The use of a variety of control oligonucleotides has been reported in different studies ranging from oligonucleotides containing 1–7 mismatches, to scrambled, sense or random sequences. When control oligonucleotides are designed, some of the motifs that cause adverse or different biological effects can be unknowingly deleted or introduced, resulting in equal or higher activity than the **antisense oligonucleotide**, which can lead to confusing results. Therefore, it might be appropriate to use more than one control oligonucleotide to establish the antisense activity depending on the sequence.

Cellular uptake facilitators for in vitro *studies*

Discussion of cellular uptake facilitators might not be relevant for *in vivo* studies, as no such agents are currently used *in vivo*, but it is certainly important in the initial screening of antisense **PS-oligonucleotides** in cell cultures and their application for functional genomics. The cellular uptake of negatively charged oligonucleotides is one of

The outstanding questions

- What is the impact of the nucleotide base composition of oligonucleotides on their antisense activity and their mechanism of action?
- What are the biological roles of various nucleotide motifs present in oligonucleotides?
- Is it possible to conclusively rule out the possibility that no mechanism other than a *bona fide* antisense mechanism is responsible for the observed biological effects of **antisense oligonucleotides** *in vivo*?
- How are oligonucleotides taken up by cells, transported and processed by various tissues?
- What is the mechanism of intestinal absorption of oligonucleotides and how can the oral bioavailability of oligonucleotides be improved?

the important factors in determining the efficacy of **antisense oligonucleotides**; the mechanism of their uptake is not yet understood in detail. *In vitro*, cellular uptake of **antisense oligonucleotides** depends on many factors, including cell type, kinetics of uptake, tissue culture conditions, and chemical nature, length and sequence of the oligonucleotide. Any one of these factors can influence the biological activity of an **antisense oligonucleotide**. It is therefore appropriate to study each **antisense oligonucleotide** in its own context, and relevant cell line, without generalizing the results for every oligonucleotide.

Cationic macromolecules such as lipofectin are used to enhance the uptake of **antisense oligonucleotides** *in vitro*. These polycations form complexes with negatively charged oligonucleotides and facilitate their internalization. Complex formation between oligonucleotides and cationic lipid agents might also provide stability against nucleases in cell cultures. The use of cell uptake facilitators could influence the outcome of the biological activity depending on the nature of the polycation used.

Concluding remarks

Many questions about the effects of **antisense oligonucleotide** sequence, secondary structures, cellular uptake, metabolism, excretion, tissue distribution, side effects and mechanism of action have been answered to a large extent, if not completely, in the past few years. As the antisense field progresses and the critical chemical and mechanistic issues of antisense effects are distinguished from those of non-antisense effects, it is becoming clear that **antisense oligonucleotide** therapeutics can in fact be as simple as complementary base recognition, but only if proper design precautions and controls are used. A number of chemical modifications have been developed and tested for antisense activity. Although a first generation **PS-oligonucleotide** has been approved for treating CMV-induced retinitis, a combination of modifications is clearly needed to fine-tune the physicochemical and biochemical properties of **antisense oligonucleotides** to make them effective drugs for multiple applications. **MBOs** have become the choice for second-generation **antisense oligonucleotides**, and several are now being tested for their potential in human clinical trials. In addition, the oral bioavailability of **MBOs** might allow these drugs to be administered in pill form in the near future[43]. The knowledge gained about the effects of antisense **PS-oligonucleotides** and their chemical modifications in the past few years is valuable for the development of antisense drugs in the future. Nonetheless, as is always the case, caution must be exerted in experimental design and interpretation of antisense results until all the critical aspects of **antisense oligonucleotides** are explored beyond reasonable doubt.

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